Carbohydrate Polymers 93 (2013) 135–143

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Heat stress causes alterations in the cell-wall polymers and anatomy of coffee leaves (*Coffea arabica* L.)

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ARTICLE INFO

Article history:
Received 3 November 2011
Received in revised form 6 March 2012
Accepted 4 May 2012
Available online 17 May 2012

Keywords:
Polysaccharides
Cell wall
Heat stress
Coffee leaves
Coffea arabica

ABSTRACT

Coffee plants were subjected to heat stress (37 °C) and compared with control plants (24 °C). Cell wall polysaccharides were extracted using water (W), EDTA (E) and 4 M NaOH (H30 and H70). In addition, monolignols were analyzed, and the leaves were observed by microscopy. Plants under heat stress accumulated higher contents of arabinose and galactose in fraction W. Xylose contents were observed to decrease in H30 fractions after the heat stress, whereas galactose and uronic acid increased. H70 fractions from plants exposed to heat stress showed increased xylose contents, whereas the contents of arabinose and glucose decreased. Differences in the molar-mass profiles of polysaccharides were also observed. The primary monolignol contents increased after the heat stress. Structural alterations in palisade cells and ultrastructural damage in chloroplasts were also observed. Our results demonstrate that the chemical profile of coffee cell-wall polymers and structural cell anatomy change under heat stress.

1. Introduction

Plant cell walls are complex, dynamic and sophisticated structures with central roles in plant form, growth and development. Cell walls also play an important role in plant defense and responses to environmental stresses (Sorensen, Domozych, & Willats, 2010). Composed of polysaccharides, proteins and phenolics, cell walls generally belong to either primary or secondary cell wall categories. Primary cell walls generate turgor pressure (thus resisting tensile forces), accommodate cell expansion, mediate cell adhesion, and occur at the surface of most plant cells. Secondary cell walls are restricted to specific types of differentiated cells, tend to be thicker than primary walls, and resist compressive forces. The primary cell walls of all plants are composed of cellulose, pectins, hemicelluloses, and protein or phenolic compounds and can yield to turgor pressure, thereby allowing expansion and growth to occur. Secondary cell walls are produced after the cessation of cell expansion in certain specialized cells. These walls represent composites of cellulose and hemicelluloses and are often impregnated with lignin (Cosgrove, 1993).

Pectins are a group of polysaccharides that contain a high proportion of 1,4-linked α-D-galactosyluronic acid residues. The pectic matrix provides an environment for the deposition, slippage and extension of the cellulosic-glycan network. Pectic polysaccharides are involved in the control of cell wall porosity and are the major adhesive material between cells (Willats, McCartney, Mackie, & Knox, 2001). The main pectic polysaccharides are homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II.

Hemicelluloses are functionally defined as polysaccharides that have β-(1 → 4)-linked backbones with an equatorial configuration. These polysaccharides form strong hydrogen-bonded complexes with cellulose fibers. Thus, the most important biological role of hemicelluloses is to strengthen the cell wall. The main hemicelluloses in primary cell walls are xyloglucans and glucuronoarabinoxylans, whereas glucuronoxylans are the main hemicelluloses in the secondary cell walls of eudicotyledons (Scheller & Ulvskov, 2010). On the other hand, lignin is often present in the secondary cell wall and is mainly composed of the monolignols *p*-coumaryl, coniferyl and sinapyl alcohols, which produce p-hydroxyphenyl H, guaiacyl G, and syringyl S monomers (or phenylpropanoid units), respectively, when incorporated into the

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0144-8617
http://dx.doi.org/10.1016/j.carbpol.2012.05.015

lignin polymer (Boerjan, Ralph, & Baucher, 2003). In leaves, cell types with non-lignified primary walls include the palisade, spongy mesophyll, and epidermal cells, whereas cell types with lignified secondary walls often include xylem tracheary elements and sclerenchyma fibers (Taiz & Zeiger, 2006).

The coffee plant is a woody perennial evergreen eudicotyledon that belongs to the Rubiaceae family (Davis, Govaerts, Bridson, & Stoffelen, 2006). Two main species of coffee are cultivated in 80 countries among four continents. These species are *Coffea arabica* L. and *Coffea canephora* Pierre, which represent 70 and 30% of world coffee production, respectively. Coffee is currently ranked second on international trade exchange markets, having a monetary value that is only surpassed by oil. The international trade of coffee generates over US$ 90 billion annually, and its production involves approximately half a million people in various processes from cultivation to the generation of the final product for consumption (Budzinski et al., 2010; Praxedes, Damatta, Loureiro, Ferrão, & Cordeiro, 2006).

Heat stress due to high ambient temperatures is a serious threat to crop production and quality worldwide. Plants are frequently subjected to heat stress, which can lead to a severe slowing of the growth and development and may even cause plant death. Extensive agricultural losses have been attributed to disturbances in growth caused by heat (Kotak et al., 2007). In recent years, this issue has become more important, due to the phenomenon of global warming. According to the Intergovernmental Panel on Climate Change, by the end of the twenty-first century, the temperature may rise between 1 and 5.8 °C in tropical areas (Jones, New, Parker, Martin, & Rigor, 1999). Heat stress can cause direct injuries to plants, such as protein denaturation and aggregation, and an increase in the fluidity of membrane lipids. Heat stress can also result in indirect injuries, such as the inactivation of enzymes in chloroplasts and mitochondria, the inhibition of biosynthesis, increased protein degradation, a loss of membrane integrity, and changes in the plant cell wall. These types of injuries eventually disturb cellular homeostasis and lead to starvation, reduced ion flux, the production of toxic compounds, and reactive oxygen species (ROS) and the inhibition of plant growth (Wahid, Gelani, Ashraf, & Foolad, 2007; Yang et al., 2006).

It has been shown that heat stress affect the growth and yield of *C. arabica* (Drinnan & Menzel, 1995) and increase the raffinose and stachyose levels during abiotic stresses (Santos et al., 2011). Although heat stress has been shown to affect oligosaccharides (Santos et al., 2011), no data have been collected on cell wall modifications associated with coffee plants under heat stress.

In this study, we evaluated how the cell wall polysaccharides of coffee leaves are changed under heat stress conditions. Furthermore, because structural variations in the lignin contents of cell walls under abiotic stress may be the result of increased oxidative stress in wall-modifying plant tissues (Moura, Bonine, Viana, Dornelas, & Mazzafera, 2010), we investigated the changes in the monolignol content of the cell walls of *C. arabica* leaves under heat stress. The leaves were also observed by microscopy in order to identify structural changes.

2. Materials and methods

2.1. Sample preparation

C. arabica cv. IAPAR-59 was cultivated under field conditions in the experimental station of the Agronomic Institute of Paraná (Londrina, Brazil). Initially, each plant was irrigated daily and fertilized weekly with 100 mL of Hoagland's nutrient solution (Hoagland & Arnon, 1938). The heat stress experiment was carried out in a growth chamber with the following parameters: 16/8 h light/dark, 45% humidity and a photosynthetic photon flux density of approximately 250 mmol m^{-2} s^{-1}. The experimental plot contained twenty two-month-old cultivated plants in 15 L pots (one plant per container) that were each filled with identical amounts of a soil, sand, and an organic compound mixture (3:1:1). These plants were grown for 7 days in a growth chamber at 24 °C. Then, leaves were harvested and considered control. Subsequently, the temperature was increased to 37 °C and leaves were harvested after three days (Day 3) and five days (Day 5). Leaves from the same pair were collected from plants at each sampling period. After harvesting, all samples were frozen immediately in liquid nitrogen after the removal of the main vein, ground to fine powders in a mortar and pestle, and stored in a freezer (−20 °C) until use.

2.2. Cell wall extraction

Lyophilized leaves were submitted to treatment according to a procedure by Albini et al. (1994) to remove chlorophyll and low-molecular-weight compounds. Polysaccharides were sequentially extracted from leaves as follows: three extractions with water (80 °C, 5 h; fraction W); two extraction with 2% EDTA (30 °C, 5 h; fraction E); three extractions with 4 M NaOH (30 °C, 5 h; fraction H30); and one extraction with 4 M NaOH (70 °C, 3 h; fraction H70). The alkaline solutions contained $NaBH_4$ to prevent end peeling. Polysaccharides were precipitated using ethanol (3:1, v/v) and stored overnight at 4 °C. The polysaccharides were subsequently isolated by centrifugation (8000 × *g*) and were washed three times with ethanol. The EDTA and 4 M NaOH fractions were dialyzed for 48 h. All fractions were submitted to enzymatic treatment for starch removal using a previously reported protocol (Bacic, Moody, & Clarke, 1986). The polysaccharides were then treated with porcine α-amylase and amyloglucosidase (2 units/mg of carbohydrate) for 8 h (40 °C) in a tris-maleate buffer containing 10 mM NaCl and 1 mM $CaCl_2$. After the digestion was complete, the polysaccharides were precipitated with ethanol (3:1, v/v), stored overnight at 4 °C and washed thoroughly with ethanol by centrifugation (8000 × *g*). The starch-free preparations were then dried under vacuum for further analyses.

2.3. Monosaccharide composition

Polysaccharides (200 μg) were hydrolyzed with 2 M TFA (0.5 mL, 5 h, 100 °C), evaporated to dryness using a Speed Vac vacuum centrifuge (Savant) and dissolved in water (0.5 mL); the monosaccharides were reduced with $NaBH_4$ (16 h at 25 °C). The resulting products were then treated with 0.5 mL of acetic acid P.A. and evaporated to dryness. After the addition of methanol (1 mL, three times), the mixture was dried, and the residue was acetylated with acetic anhydride (30 min at 100 °C) (Vinogradov & Wasser, 2005).

The resulting alditol acetates were analyzed by gas–liquid chromatography using a model 5890 S II Hewlett-Packard gas chromatograph at 220 °C (flame ionization detector and injector temperature, 250 °C) with a DB-210 capillary column (0.25 mm internal diameter × 30 m) at a film thickness 0.25 μm. Nitrogen was used as the carrier gas (2.0 mL min^{-1}).

Uronic acid content was estimated by the meta-hydroxydiphenyl colorimetric method (Blumenkrantz & Asboe-Hansen, 1973) using galacturonic acid as a standard. Insoluble fractions were determined after dissolving the samples in sulfuric acid (Ahmed & Labavitch, 1977).

2.4. High pressure size exclusion chromatography (HPSEC) analysis

The Waters high performance size exclusion chromatography (HPSEC) apparatus used was coupled to a Waters 2410

differential refractometer (RI) detector. Four Waters Ultrahydrogel 2000/500/250/120 columns were connected in series and coupled to the multidetection instrument. A solution of 0.1 M $NaNO_2$ containing NaN_3 (0.5 g L^{-1}) was used as the eluent at a flux of 0.6 mL min^{-1}. Previously filtered samples (0.20 μm; Millipore) were analyzed at 1.0 mg mL^{-1}, and data were collected and processed using the Wyatt Technology ASTRA program.

2.5. Fourier transform infrared spectroscopy (FTIR)

Samples were dried in an Abderhalden apparatus and stored in desiccators prior to FTIR analysis. Pellets were prepared from a mixture of sample and KBr in a 1:100 (w/w) ratio. The infrared spectra were collected on a Bomem Hartmann & Braun spectrometer over the range of 1800–900 cm^{-1} at a resolution of 4 cm^{-1} in the absorbance mode and were averaged using 32 scans.

2.6. Electron and optical microscopy

Leaves were fixed with modified Karnovsky's fixative (without calcium chloride and with 2.5% glutaraldehyde) (Karnovsky, 1965), washed in 0.1 M cacodylic acid buffer (pH 7.2), and fixed in 2% OsO_4 in 0.1 M cacodylic acid buffer (pH 7.3) for 1 h. Subsequently, the leaves were dehydrated with ethanol and acetone, embedded in Epon 812 (Luft, 1961), contrasted using uranyl acetate and lead citrate, and examined with a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 kV (Peabody, MA, USA). The leaf blades were fixed with Karnovsky, embedded in metacrilatoaglicol (JB-4) and sectioned in a rotary microtome. Transverse sections were stained with 0.05% toluidine blue (Feder & O'Brien, 1968), mounted in synthetic resin (Entellan®), and observed and photographed under an OLYMPUS BH-2 optical microscope.

2.7. Monolignol compositions

Alkaline nitrobenzene oxidation was used to determine the composition of monomeric lignins (Zanardo, Lima, Ferrarese, Bubna, & Ferrarese-Filho, 2009) for the final residue (20 mg). The samples were sealed in a Pyrex® ampule containing 1 mL of nitrobenzene and heated to 170 °C for 90 min; the sample was occasionally shaken during the course of the reaction. The sample was cooled at room temperature, washed twice with chloroform, acidified to pH 2 with 2 M HCl, and extracted twice with chloroform. The organic extracts were combined, dried, resuspended in 1 mL of methanol, and diluted in a mixture of methanol and 4% acetic acid in water (20:80, v/v). All of the samples were filtered through a 0.45 μm disposable syringe filter and analyzed by high performance liquid chromatography. The mobile phase consisted of a mixture of methanol and 4% acetic acid in water (20:80, v/v); a flow rate of 1.2 mL min^{-1} was used for an isocratic run of 20 min. Quantification of the monomeric aldehyde products (*p*-hydroxybenzaldehyde, vanillin and syringaldehyde) released by nitrobenzene oxidation was performed at 290 nm using the corresponding standards. Results were expressed as μg monomer mg^{-1} final residue.

2.8. Statistical design

The experimental design was completely randomized, and the experiment was represented by at least 5 plants. Biological samples were represented by pools of coffee plant leaves collected at the same developmental stage. The data are expressed as the mean of three independent experiments ± S.E. A one-way variance analysis for testing the significance of the observed differences was performed using Prisma® software (Version 5.0). In addition, the differences between parameters were evaluated using the Tukey test; p values ≤ 0.05 were considered to be statistically significant.

Table 1
Yields[a] of cell wall fractions from *C. arabica* leaves maintained at 24 °C (control) and after 3 (Day 3) and 5 (Day 5) days under heat stress at 37 °C.

Days/fraction	Pectins		Hemicelluloses		Cellulose
	W (%)	E (%)	H30 (%)	H70 (%)	R (%)
Control	10.3	4.8	16.7	2.9	18.4
Day 3	5.3	4.5	25.3	3.2	17.0
Day 5	4.8	3.2	22.7	2.8	16.6

[a] Expressed as percentages of the total leaf based on the insoluble residues obtained through sequential treatment with chloroform and methanol:water/7:3.

3. Results and discussion

3.1. Changes in polysaccharide yields

The yields of polysaccharides from the sequential extraction of cell walls under abiotic stress can provide important information regarding alterations in the cell wall composition (Konno, Yamasaki, Sugimoto, & Takeda, 2008). The polysaccharides from coffee leaves that have been subjected to heat stress were extracted using water (fraction W), EDTA (fraction E), and 4 M NaOH (H30 and H70 fractions). The yields of each fraction were compared with those of the control group (Table 1). The hemicellulosic fractions (H30 and H70) were the most abundant fractions, whereas the pectin contents (W and E fractions) were the smallest fractions. Heat stress resulted in an increase in leaf hemicellulosic content, whereas the pectin content decreased by almost 50% compared to the control plants. On the other hand, the final insoluble residue yields were not affected by 5 days of heat stress treatment. In cultured tobacco cells (*Nicotiana tabacum* L.) subjected to osmotic stress, an increase in hemicellulosic fractions, resulting in increased cell rigidity and a loss of growth capacity, has been observed (Iraki, Singh, Bressan, & Carpita, 1989; Muñoz, Dopico, & Labrador, 1993). Our observation of a decrease in the proportion of pectins extracted from heat-stressed coffee leaves using hot water and EDTA (Table 1) may indicate that the treatment changed the organization of the pectins, which has been previously proposed for tobacco cells subjected to saline and osmotic stresses (Iraki, Bressan, Hasegawa, & Carpita, 1989).

3.2. Changes in water-soluble polysaccharides

The determination of uronic acid and neutral monosaccharide contents indicated that arabinose and galactose were the most abundant components of fraction W (Fig. 1A), thereby suggesting the presence of arabinogalactans. Type I arabinogalactans are typically associated with pectins, whereas type II arabinogalactans are associated with proteins being called arabinogalactan-proteins. Type I arabinogalactans has previously been extracted with hot water from eudicotyledon leaves (Duan, Zheng, Dong, & Fang, 2004). In addition, type II arabinogalactan has been isolated via a hot-water extraction from coffee beans (Capek, Matulová, Navarini, & Suggi-Liverani, 2010). The FTIR spectrum of fraction W confirmed the presence of type II arabinogalactan (Fig. 1C), which were indicated as bands at 1139, 1078, and 1043 cm^{-1} (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000).

As expected, uronic acid and rhamnose, which are derived from homogalacturonans and rhamnogalacturonans, were found as components of fraction W. Glucose, mannose, xylose and minor amounts of fucose were also detected. The occurrence of mannose and glucose could have been related to the presence of glucomannans, which have been extracted from the *Ginkgo biloba* leaf (Yang,

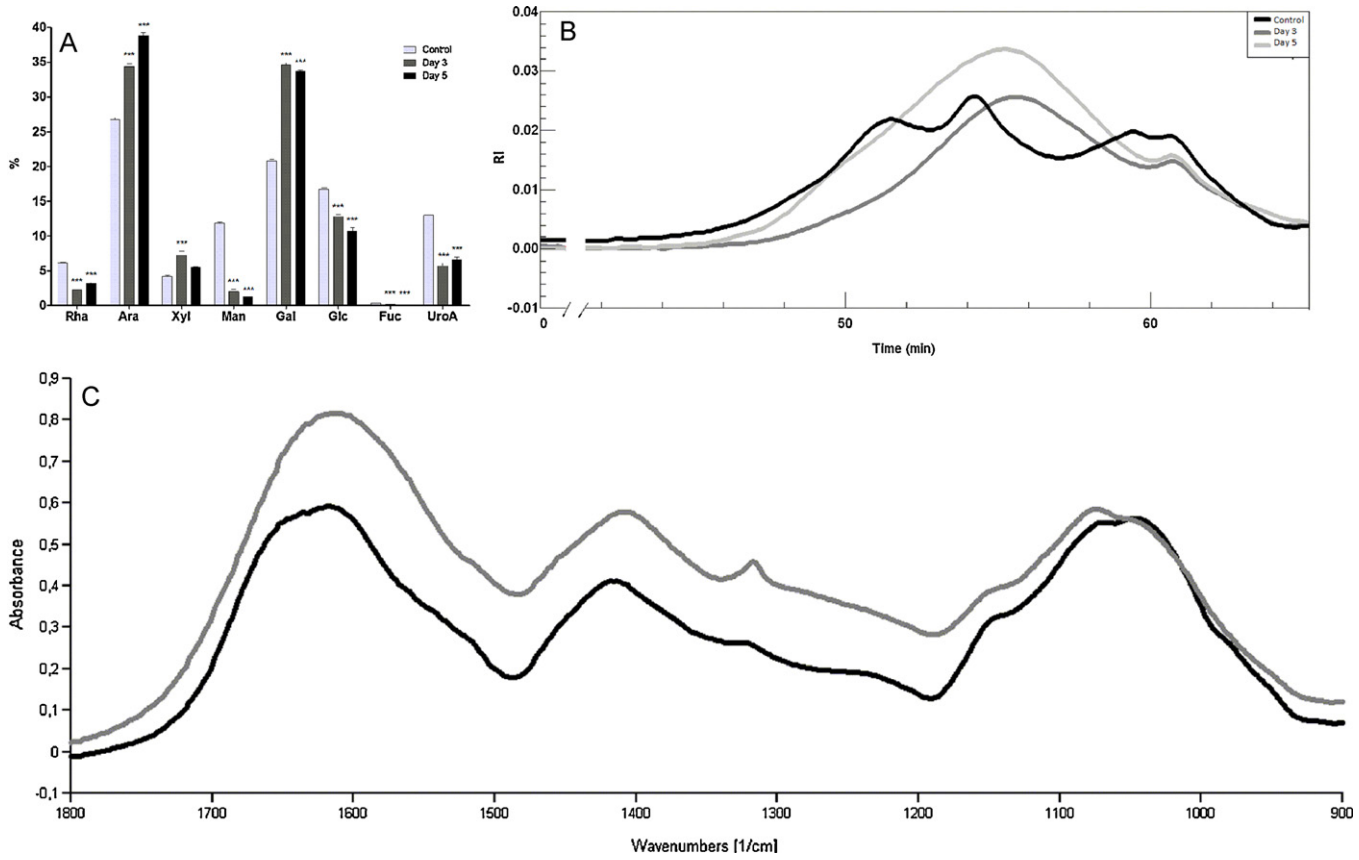

Fig. 1. Monosaccharide composition (A), HPSEC analysis with RI detection (B) and the FTIR spectra (C) of W fractions from *C. arabica* leaves maintained at 24 °C (control) and after 3 (Day 3) and 5 (Day 5) days under heat stress at 37 °C. Error bars indicate the SE. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Zhou, & Liang, 2009) and *Salvia officinalis* (Capek, 2009) using hot water, even though glucomannans are a type of hemicellulose. Xylose may have been derived from xylogalacturonan, which has been identified in the leaf of *Diospyros kaki* (Duan, Wang, Dong, Fang, & Li, 2003).

After heat stress, the arabinose and galactose contents increased, whereas the mannose, glucose, uronic acid, rhamnose and fucose contents decreased. The FTIR spectrum of fraction W isolated from plants subjected to heat stress showed more pronounced bands from arabinogalactan in addition to bands between 1600 and 1500 cm^{-1} that correspond to proteins (Kačuráková et al., 1999) (Fig. 1C). The increase in arabinose and galactose contents might indicate response of coffee leaves to heat stress related to type II arabinogalactans. Type II arabinogalactans are implicated in many biological processes of cell proliferation and survival. It has been proposed that type II arabinogalactan can be evolved in the rigidification of cell wall by oxidative crosslinking (Seifert & Roberts, 2007). Moreover, an increase in type II arabinogalactan amounts might be related to increased water-holding capacity (Fincher & Stone, 1983).

Results reported by Moore et al. (2006) studying the response of the leaf cell wall to desiccation in the resurrection plants were similar to the results find in this work for coffee leaves subjected to heat stress. Moore et al. (2006) detected significant differences between hydrated and desiccated states in arabinogalactan content. According to these authors, arabinan-rich leaf cell wall in resurrection plants contributes to the ability of the plant to survive when undergo repeated periods of desiccation and rehydration.

The decrease in rhamnose and uronic acid contents of plants subjected to heat stress is in agreement with the hypothesis proposed by Iraki et al. (1989a) that the exposure to heat stress changes the organization of cell wall pectins in plant cells under saline and osmotic stress. It is possible that under heat-stress conditions, the association between pectic polysaccharides become stronger, making them less extractable. The data not allow to state if structural changes took place in the pectic polysaccharides under stress conditions, however this possibility can not be ruled out.

Cell-wall porosity is regulated by pectins (Baron-Epel, Gharyal, & Schindler, 1988). A decrease in pectin content could result in larger pores, which could contribute to increased conductive heat loss by evaporation. An opposite effect has been reported for plants under freezing stress (Ashworth & Abeles, 1984).

Elution profiles obtained by HPSEC using the RI detector showed pronounced differences between the fractions under heat stress conditions compared to those of the control group (Fig. 1B). For the control plants, four main peaks could be identified with elution times of approximately 51, 54, 59 and 61 min. However, for plants subjected to heat stress, only two peaks were identified: a main peak eluted at approximately 55 min, and a second peak at approximately 61 min, which coincides with the lower molar mass polymers from the control group. Heat stress that results in increased H_2O_2 levels (Wahid et al., 2007) could contribute to an increase in the cross-linking between cell wall polymers, thereby altering the mass-average molar mass of the polysaccharides. It has been proposed that type II arabinogalactan participate in the modulation of cell wall mechanics under stress conditions via the rigidification of the cell wall by oxidative crosslinking (Seifert & Roberts, 2007). These changes may cause a decrease in cell wall extensibility and prevent wall-stress relaxation.

The possible cell wall modifications observed for plants under heat stress were consistent with those observed in plant cells under osmotic or water stress (Iraki et al., 1989a,b; Renault & Zwiazek,

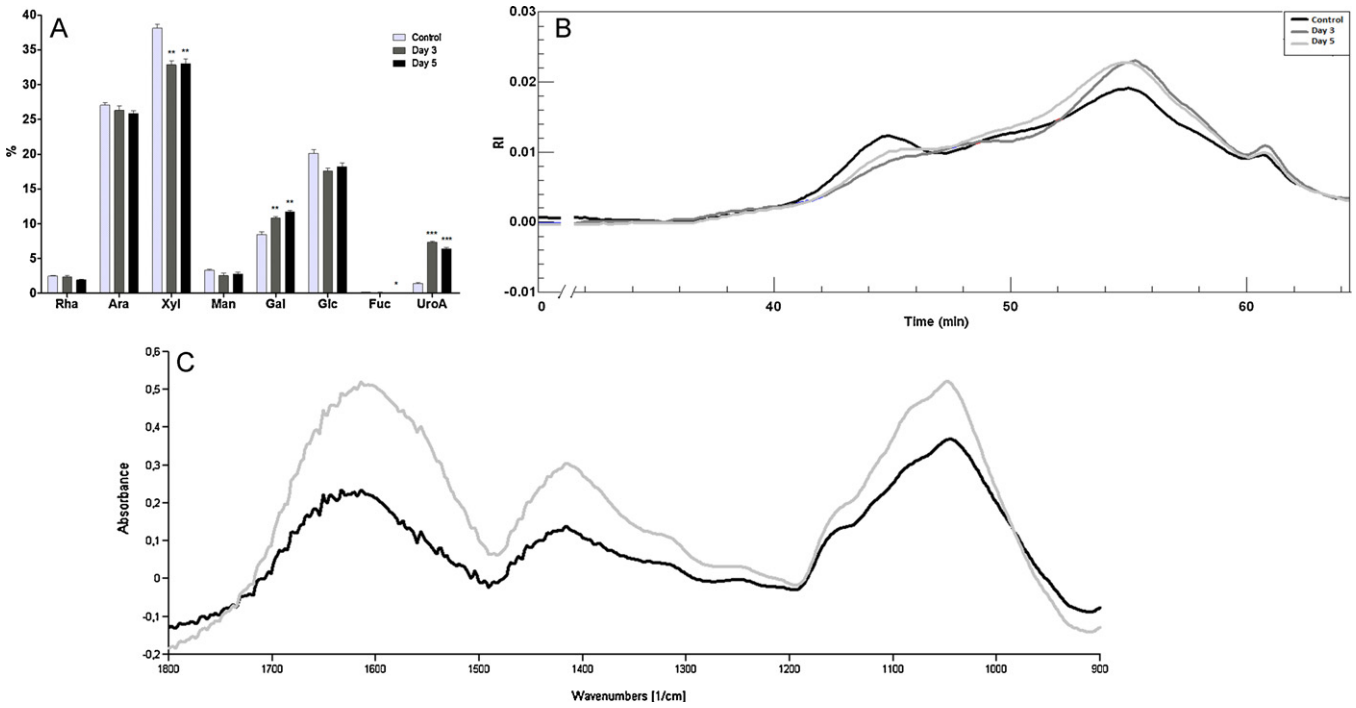

Fig. 2. Monosaccharide composition (A), HPSEC analysis with RI detection (B) and FTIR spectra (C) of H30 fractions from leaves of *C. arabica* control (24 °C) and leaves subjected to 3 and 5 days of heat stress (37 °C). Each value represents the mean of three determinations. Error bars indicate the SE. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

1997; Zwiazek, 1991). Thus, our results may indicate a similar role for cell walls in plant modifications in response to water or heat stresses.

3.3. Changes in the hemicellulosic fractions

The composition of monosaccharides in hemicellulosic fractions (Figs. 2A and 3B) showed xylose and arabinose as the main components of the H30 and H70 fractions, suggesting the presence of arabinoxylans. These results are in agreement those obtained by Cecy and Corrêa (1984), who reported the presence of arabinoxylans in the leaves of *C. arabica* L. var *Mundo Novo*. Wenzel and Corrêa (1977) also identified a 4-*O*-methyl-glucuronoxylan in the hemicellulosic fraction from the leaves of *C. arabica* L. var *Mundo Novo*. Also, 4-*O*-methyl-glucuronoxylan has been reported to be the most important hemicellulose of secondary walls in fibers and vessel elements of eudicotyledons plants (York & Oneill, 2008). FTIR of the H30 and H70 fractions (Figs. 2C and 3C) showed bands related to these polymers (Kačuráková et al., 2000).

Glucose, galactose and mannose were present in higher amounts in fraction H30, whereas higher percentages of rhamnose and uronic acid were observed in fraction H70. Small amounts of fucose, probably from xyloglucans, were also detected. According to the current model of the primary cell walls of eudicotyledons, the main hemicellulose is xyloglucan with lower amounts of glucuronoarabinoxylans, glucomannans, galactoglucomannans, and galactomannans. The FTIR spectrum in Fig. 2C confirmed the presence of bands from xyloglucans (Kačuráková et al., 1999).

Under heat-stress conditions, the xylose content decreased in H30 fractions, whereas the amounts of galactose and uronic acids increased such that they were significantly different compared to control plants. The results indicated that heat stress decreased the amount of xylans extracted at 30 °C, which may have been due to the increase in the association between cell wall polymers under heat stress. The results are similar to those described by Moore et al. (2006) for the response of the leaf cell wall to desiccation in the resurrection plant. These authors observed that plants under stress, desiccated plants, had lower amounts of more soluble arabinoxylans than the hydrated plants.

The results obtained for coffee are different from those reported by Zhang, Liu, Chang and Anyia (2010). They observed that high temperature increased the arabinoxylan concentration (especially the more soluble arabinoxylans) in wheat. According to these authors, the arabinoxylans were positively correlated with water use efficiency suggesting that arabinoxylans can be increased by selecting for increased water use efficiency. However, in other report high temperature and drought stress decreased the arabinoxylans concentration in wheat. Since the variation in arabinoxylans due to genotype and environment are not fully understood, the inconsistency of the results was attributed to the differences in varieties and locations Zhang et al. (2010).

The differences in the responses observed for coffee and wheat could be due to the different patterns of cell wall, since coffee has Type I primary cell wall while wheat has Type II primary cell wall.

The increase in the uronic acid content observed in fractions from plants subjected to heat stress was also in agreement with the hypothesis that changes in the organization of cell wall polysaccharides occur in plants under heat stress. Furthermore, pectins were not extracted by hot water, due to stronger interactions with cell wall polymers. However, these compounds could be extracted under harsher extraction conditions.

HPSEC elution profiles of the H30 fractions are shown in Fig. 2B. Three main peaks with elution times of approximately 44, 55, and 61 min were detected by IR for fractions from control and stressed plants. However, several differences in the relative proportion of the peaks were observed when comparing fractions from plants under heat stress with those of the control-group plants.

The monosaccharide compositions of the H70 fractions are shown in Fig. 3A. The qualitative profile of fraction H70 was similar to that obtained for H30. However, differences were observed in the monosaccharide proportions. For example, the H70 fractions showed higher amounts of rhamnose and uronic acid, indicating the extraction of higher amounts of pectins.

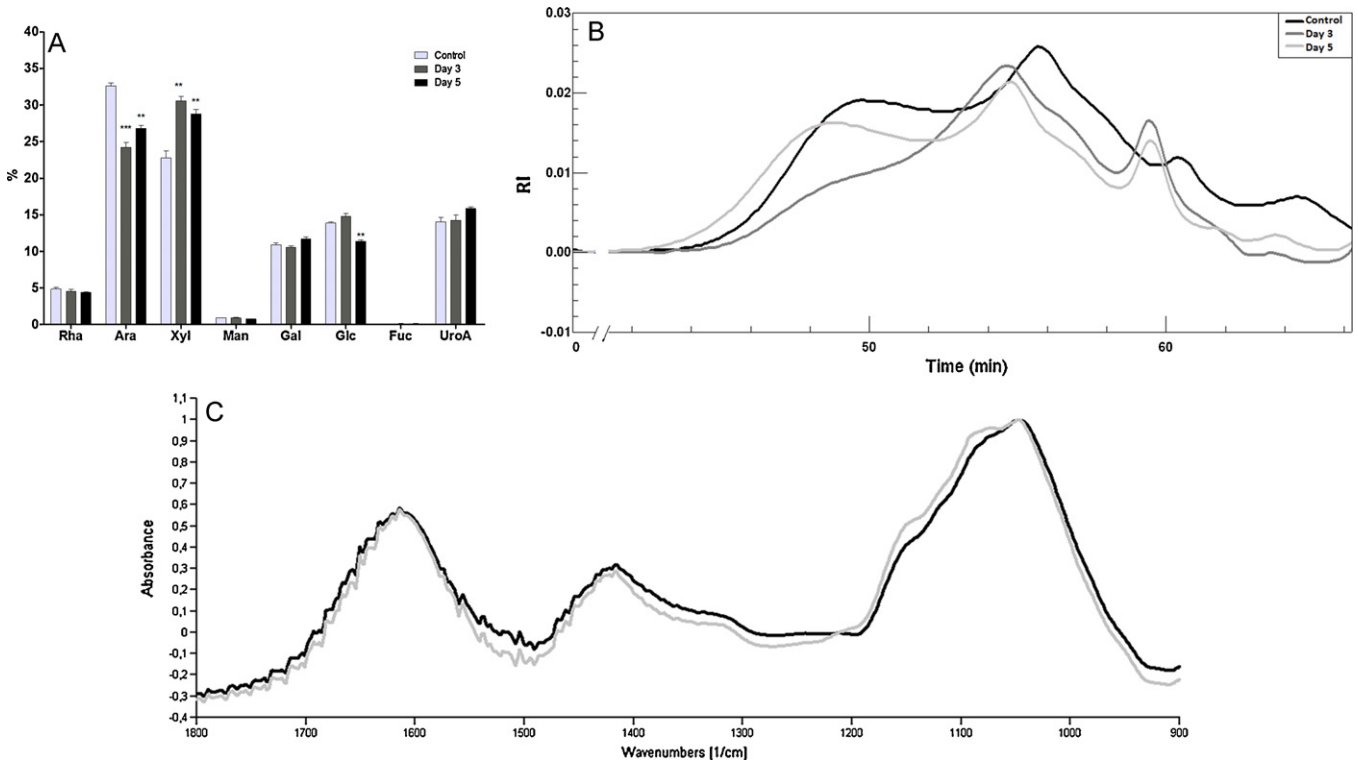

Fig. 3. Monosaccharide composition (A), HPSEC analysis with RI detection (B) and FTIR spectra (C) of H70 fractions from leaves of *C. arabica* control (24 °C) and leaves subjected to 3 and 5 days of heat stress (37 °C). Each value represents the mean of three determinations. Error bars indicate the SE. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Compared with the control, the H70 fractions extracted from plants exposed to heat stress showed increased xylose contents, whereas the arabinose and glucose contents decreased. However, there were no observed differences between the uronic acids, rhamnose, mannose, and fucose contents of the control and stressed plants.

Moore et al. (2006) also found few differences between hemicelluloses isolated under sequential extraction from hydrated and desiccated leaves of resurrection plants.

A strong, heated alkaline solution may extract hemicellulose that is tightly associated with cellulose. The observed increase in xylose content associated with a decrease in arabinose content could have been related to changes in the crosslinking of the arabinoxylans backbone with fibers of cellulose. Ara*f* substitutions are thought to hinder hydrogen-bonding between arabinoxylan molecules or cellulose microfibrils, rendering the molecule more soluble in water (Urahara et al., 2004). Type II primary cells that are used in primary wall development have been reported to have a lower extent of Ara*f* substitutions in the xylan backbone, enabling the unsubstituted xylan regions to hydrogen-bond and thus crosslink the cell wall (Carpita, 1996; Urahara et al., 2004).

The HPSEC elution profiles of the H70 fractions are given in Fig. 3B. For the sample from control plants, four peaks were identified with elution times of approximately 49, 56, 61 and 65 min. In the H70 fractions from plants subjected to heat stress, the three final peaks were observed to be shifted to have shorter elution times, indicating a higher molar mass. These results corroborate the changes observed in the monosaccharide composition results. The peak corresponding to a higher molar mass component in the H70 fraction, isolated from plants after five days of being subjected

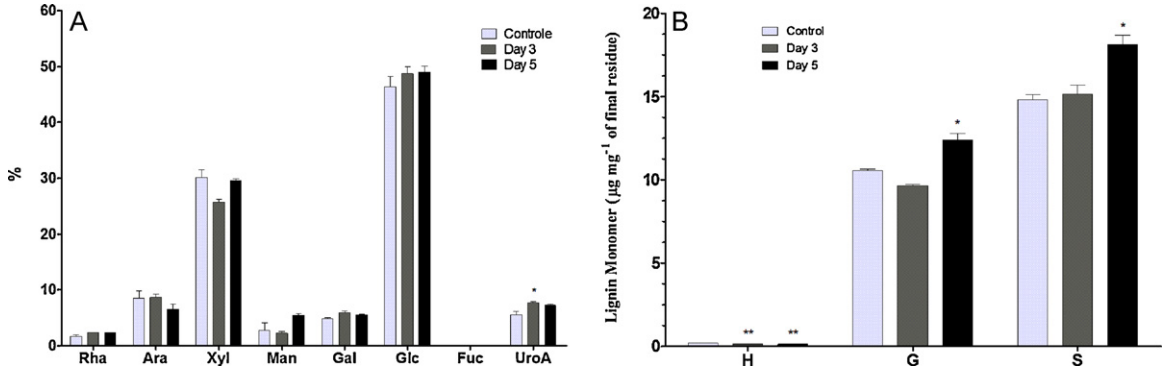

Fig. 4. Monosaccharide composition (A) and lignin monomer composition (B) of insoluble final residues from leaves of *C. arabica* control (24 °C) and leaves subjected to 3 and 5 days of heat stress (37 °C). Each value represents the mean of three determinations. Error bars indicate the SE. H, *p*-hydroxyphenyl; G, guaiacyl; and S, syringyl monomers. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

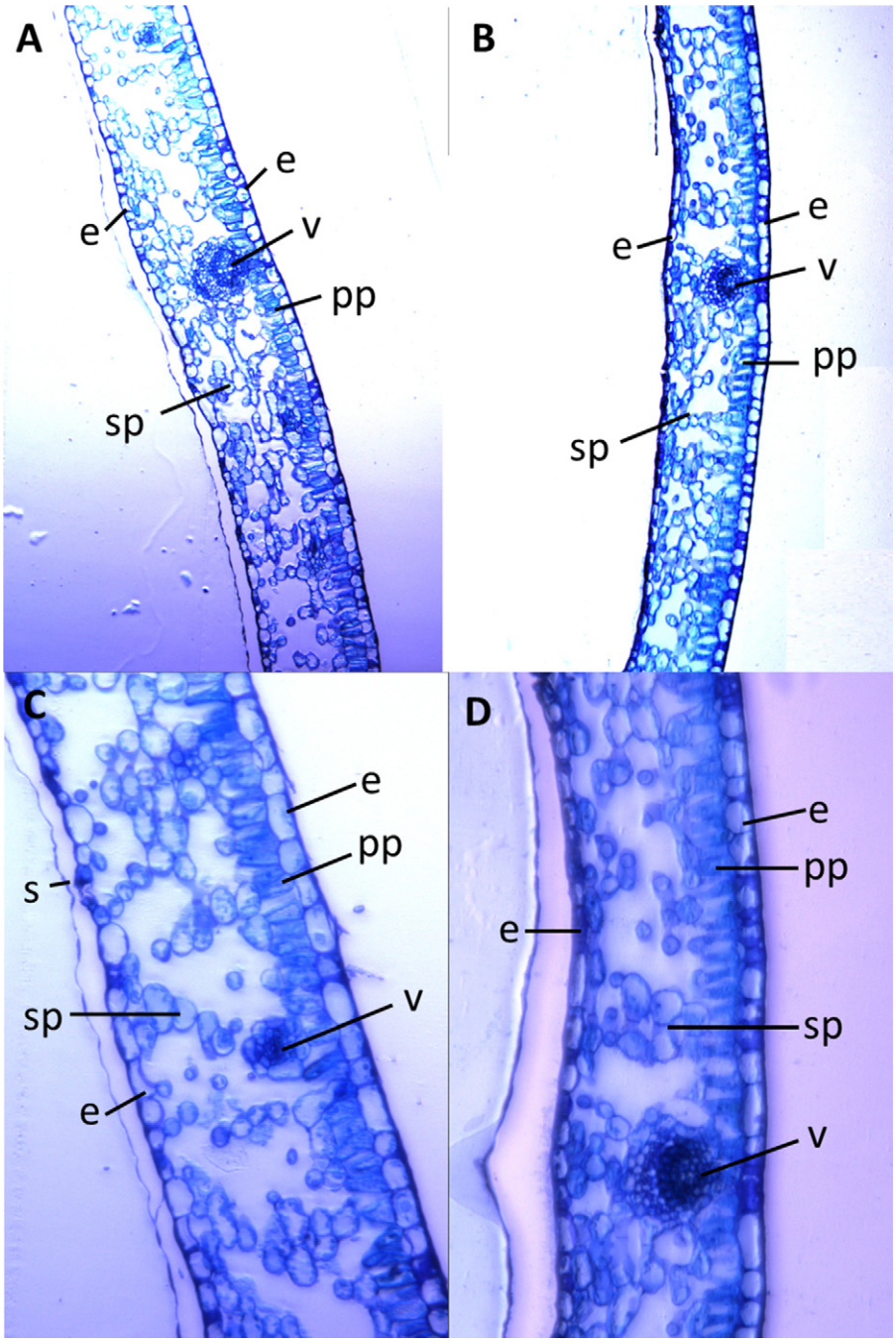

Fig. 5. The microscopic structure of the following *C. arabica* leaves: control (100×) (A); five days-stressed (100×) (B); control (200×) (C); and five days-stressed (200×) (D). Epidermis (e); vessels (xylem and phloem) (v); spongy parenchyma (sp); palisade parenchyma (pp); stomata (s).

to heat stress, was also shifted to have a shorter elution time. However, this trend was not observed for the H70 fraction isolated from plants subjected to three days of heat stress.

Few data were found in the literature concerning the changes in the molar mass elution profiles of polysaccharides obtained from plants subjected to stress conditions. Zhong & Läuchli (1993) observed changes in cell wall polymer size from the root tip of cotton seedlings grown in saline environments. In this case, authors detected an increase in the amount of hemicelluloses of higher molar mass. Iraki et al. (1989a) analyzed the extracellular polysaccharides of tobacco cell cultures and changes associated with osmotic stress and also observed changes in the elution profiles of polysaccharides after osmotic stress.

3.4. Changes in the final insoluble residue

As expected, the insoluble final residues were rich in glucose arising from cellulose (Fig. 4A). However, the final residues had a high content of non-cellulosic monosaccharides, the most abundant of which was xylose followed by arabinose, uronic acids, galactose, mannose, rhamnose, and traces of fucose. According to Edelmann and Fry (1992), the commonly used 4 M alkali is not completely effective for the extraction of hemicelluloses, which requires 6 M NaOH.

The presence of the secondary cell wall in the final insoluble residues was confirmed by the analyses of monolignols from lignin (Fig. 4B). The final insoluble residues obtained after the sequential extraction of coffee leaves exhibited high G and S monolignol

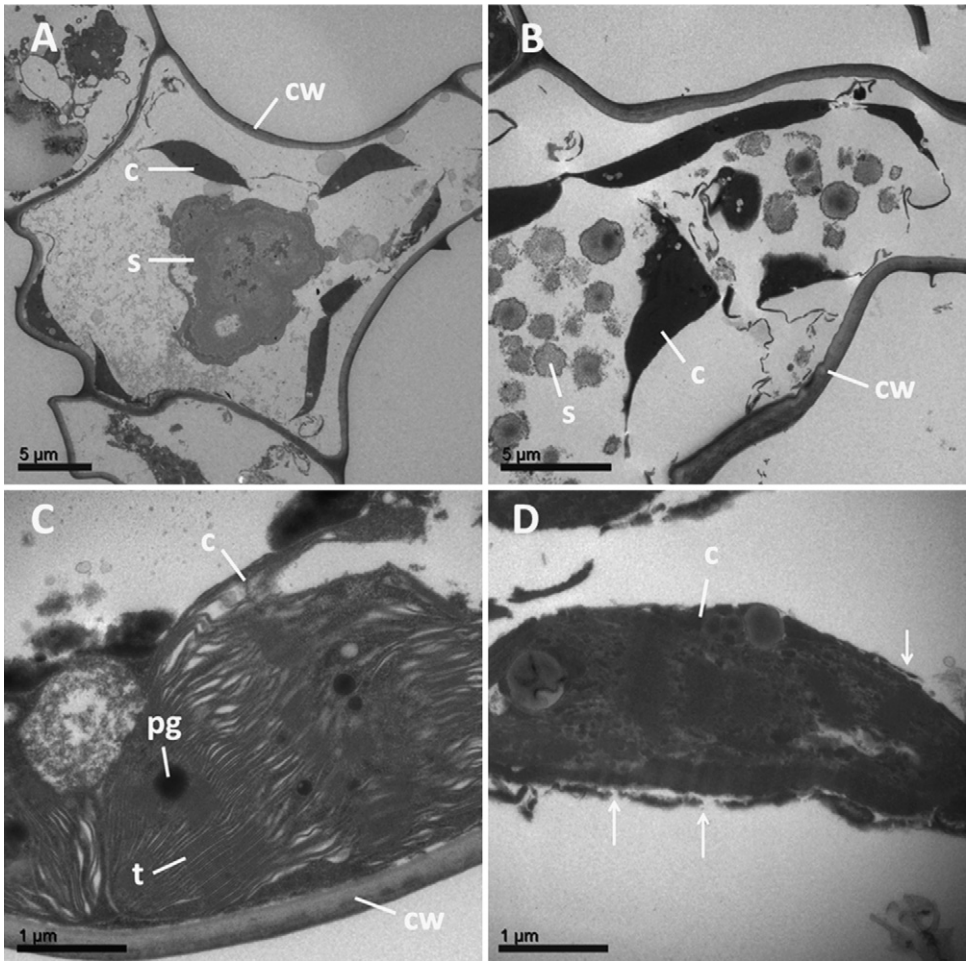

Fig. 6. Transmission electron microscopy images of mesophyll cells from *C. arabica* leaves: Control cell (A); cell under heat stress (B); chloroplast of control (C); and chloroplast of cell under heat stress (D). Cell wall (cw); mitochondria (m); plastoglobuli (pg); chloroplast (c); thylakoids (t); starch (s).

contents, which mainly occur in angiosperms (Boerjan et al., 2003). Under heat stress, increases in the G and S monolignol contents were observed, whereas a decrease in the H monolignol content was seen. This increase in the monolignol content is in agreement with the results obtained for the leaves of strawberry plants (*Fragaria ananassa*) subjected to heat stress (Gulen & Eris, 2004). Lignin polymerization has been shown to require peroxidases or laccases plus H_2O_2 (Moura et al., 2010). Furthermore, heat stress has been reported to cause the production of ROS such as H_2O_2, in plants (Suzuki & Mitller, 2006) and could generate H_2O_2 in the coffee leaf, thereby stimulating monolignol polymerization.

3.5. Structural changes

The mesophyll of *C. arabica* is organized as one layer consisting mainly of elongated columnar palisade parenchyma and a smaller proportion of spongy parenchyma cells that are irregularly shaped, thereby allowing gases to circulate through abundant air spaces (Fig. 5A and C). However, after five days of heat stress, palisade parenchyma cells were observed to be more separated and thinner relative to control. Consequently, the total thickness of the leaves was also thinner (Fig. 5B and D).

Ultrastructural microscopy showed that the control mesophyll cells had large amounts of starch in the cytoplasm, whereas in five days-stressed cells, the fragmentation of starch was observed (Fig. 6A–D). Moreover, in the control, chloroplasts were organized and contained a plastoglobuli reserve. After five days under stress, the membranes and thylakoids were damaged with a loss of grana stacking. These types of effects have been observed in leaves of other plants (Djanaguiraman, Prasad, Boyle, & Schapaugh, 2011; Zhang, Chen, Zhang, Zheng, & Liu, 2009; Zhang et al., 2010).

Collectively, the results of this study indicate that heat stress cause modifications in the cell wall and structure of the cell mesophyll. These results support the hypothesis that plants undergo cell-wall reestablishment, which involves the biosynthesis or assembly of major cell-wall compounds (Ko & Han, 2004) and damages leaf cells.

4. Conclusions

The results obtained here indicate that heat stress cause modifications in the cell wall of the coffee leaf. The content, composition and molar mass profiles of polysaccharides from the cell wall showed differences compared relative to the control. These results suggest that the exposure of coffee leaves to heat stress changes the organization of cell-wall polysaccharides, as proposed in previous work on plants subjected to other stress conditions. After the removal of soluble cell-wall polysaccharides from leaves under heat stress, the monolignol contents of the final residues increased. Structural alterations in palisade cells and ultrastructural damage in chloroplasts were also observed in plants under heat stress.

Acknowledgments

We thank the Brazilian agencies CNPq, CAPES and Fundação Araucária for providing financial assistance.

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